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Colistin MIC Variability by Method for Contemporary Clinical Isolates of Multidrug-Resistant Gram-Negative Bacilli

Janet A. Hindler, Romney M. Humphries

Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California, USA

***In vitro* evaluation of colistin susceptibility is fraught with complications, due in part to the inherent cationic properties of colistin. In addition, no reference method has been defined against which to compare the results of colistin susceptibility testing. This study systematically evaluated the available methods for colistin MIC testing in two phases. In phase I, colistin MICs were determined in 107 fresh clinical isolates of multidrug-resistant (MDR) Gram-negative bacilli (GNB) by broth microdilution with polysorbate 80 (BMD-T), broth macrodilution (TDS), and the Etest. In phase II, 50 of these isolates, 10 of which were colistin resistant, were tested in parallel using BMD-T, TDS, agar dilution, broth microdilution without polysorbate 80 (BMD), and the TREK Gram-negative extra MIC format (GNXF) Sensititre. The Etest was also performed on these 50 isolates using Mueller-Hinton agar (MHA) from three different manufacturers. Colistin MIC results obtained from the five methods were compared to the MIC results obtained using BMD-T, the method that enables the highest nominal concentration of colistin in the test medium. Essential agreement ranged from 34% (BMD) to 83% (TDS), whereas categorical agreement was >90% for all methods except for BMD, which was 88%. Very major errors (VMEs) (i.e., false susceptibility) for the Etest were found in 47 to 53% of the resistant isolates, depending on the manufacturer of the MHA that was used. In contrast, VMEs were found for 10% ($n = 1$) of the resistant isolates by BMD and 0% of the isolates by the TDS, agar dilution, and Sensititre methods. Based on these data, we urge clinical laboratories to be aware of the variable results that can occur when using different methods for colistin MIC testing and, in particular, to use caution with the Etest.**

Increased antimicrobial resistance among clinically important Gram-negative bacilli (GNB) has renewed interest in colistin as a therapeutic option (1). Currently, there are no FDA-cleared *in vitro* tests for colistin. Many clinical laboratories use disk diffusion (DD), Etest (bioMérieux, Durham, NC), or TREK Sensititre (TREK Diagnostic Systems, Inc., Cleveland, OH) methods for testing colistin; the latter two are labeled as research use only (RUO) in the United States. It has long been appreciated that colistin DD is problematic for nonpseudomonal isolates (2, 3) and yields high rates of very major errors (VMEs) (i.e., false susceptibility) compared to with the use of reference agar dilution. More recent data suggest that this is also true for contemporary *Pseudomonas aeruginosa* isolates. Up to 32% of VMEs have been observed by DD testing compared to reference MICs obtained using either broth or agar dilution reference methods (4–8). The Clinical and Laboratory Standards Institute (CLSI) provides DD breakpoints for *P. aeruginosa* (9) but not for any other GNB, and one manufacturer of colistin disks (BBL, BD Diagnostic Systems, Sparks, MD) suggests that any DD zone in the susceptible range be confirmed by a method that yields an MIC (Sensi-Disc antimicrobial susceptibility test disc package insert, no. 8840621 2010/07; BD BBL). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) does not provide any colistin DD breakpoints in their breakpoint tables (version 3.0; http://www.eucast.org/clinical_breakpoints/). In addition to the unreliable results obtained from colistin DD tests, the reliability of colistin MICs obtained by the Etest is a concern (6, 10), as up to 32% of VMEs compared to agar dilution (AD) have been observed in some studies.

Thus, the dilemma of how to best perform colistin susceptibility testing is a pressing concern for clinical laboratories. This problem is complicated by the lack of a reliable reference standard method against which to compare commercial tests. Many published studies have employed AD as a standard, but this has been used infrequently by the CLSI in recent years as a reference method. This leaves broth microdilution (BMD) as the primary reference method by which to perform colistin MIC testing. However, colistin readily adheres to the plastics used for BMD panels, an effect that is most apparent at low concentrations of the drug (11). The adsorption of colistin to polystyrene can be mitigated by the addition of a surfactant, such as polysorbate 80 (e.g., Tween 80), to either the bacterial inoculum suspension or directly to the cation-adjusted Mueller-Hinton broth (CAMHB) dispensed in the wells of the BMD panels (K. Sei, presented at the meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing, Tempe, AZ, January 2012). However, the CLSI reference BMD method currently does not stipulate the use of surfactant for colistin testing (12). In addition, the surface charge on the polystyrene microplate applied during manufacturing influences the level of colistin adsorption to the plate surface (13). As the CLSI reference BMD method does not address differences in the panel plastics nor the treatment of these plastics, significant variability might exist even between laboratories performing the reference BMD method.

The purpose of this study was to systematically evaluate the available methods for colistin susceptibility testing using a collection of contemporary multidrug-resistant (MDR) GNB, isolated in 2010 and 2011 for which a colistin susceptibility test was ordered to aid in clinical decision-making.

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Address correspondence to Romney M. Humphries, rhumphries@mednet.ucla.edu.

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TABLE 1 Summary of colistin susceptibility test methods used in phase I and phase II of the study

Test[a]	Method reference	Description	Test medium[b] (manufacturer)	Inoculum[c]	Study phase
AD	CLSI M07 (12)	In-house prepared AD plates	MHA (BBL)	0.5 McFarland suspension diluted in sterile saline to obtain 10^4 CFU/spot	II
BMD	CLSI M07 (12)	In-house prepared BMD panels in untreated polystyrene microplates	CAMHB (Difco)	0.5 McFarland suspension diluted in sterile water to obtain 3×10^5 to 5×10^5 CFU/ml	II
BMD-T	Modification of CLSI M07 (12)	In-house prepared BMD panels in untreated polystyrene microplates	CAMHB (Difco)	0.5 McFarland suspension diluted in sterile water + 0.02% polysorbate 80 to obtain 3×10^5 to 5×10^5 CFU/ml; final polysorbate 80 concn, 0.002%	I & II
TDS	CLSI M07 (12)	In-house prepared tube dilution in borosilicate tubes washed with Micro-90	CAMHB (Difco)	0.5 McFarland suspension diluted in CAMHB to obtain 3×10^5 to 5×10^5 CFU/ml	I
Etest	bioMérieux package insert	Agar gradient diffusion	MHA (BBL); MHA (Remel); MHA (Hardy)	0.5 McFarland suspension	I
TREK GNXF Sensititre panels	TREK package insert	Dried MIC panel	Sensititre cation-adjusted Mueller-Hinton broth with TES buffer	0.5 McFarland suspension diluted in deionized water to obtain 3×10^5 to 5×10^5 CFU/ml	II

[a] AD, agar dilution; BMD, broth microdilution; BMD-T, broth microdilution with 0.002% polysorbate 80; TDS, broth macrotube dilution.
[b] MHA, Mueller-Hinton agar; CAMHB, cation-adjusted Mueller-Hinton broth; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
[c] The initial suspension of the organism was prepared in normal saline for all testing, with the exception of TREK GNXF, for which the suspension was prepared in deionized water.

MATERIALS AND METHODS

Bacterial isolates. One hundred seven GNB isolated from unique patients for which a colistin MIC test was ordered by an infectious diseases specialist between January 2010 and January 2011 were used in this study. The isolates comprised *P. aeruginosa* ($n = 60$), *Klebsiella pneumoniae* ($n = 20$), and *Acinetobacter baumannii* ($n = 27$). All isolates were classified as MDR (e.g., resistant to at least one antimicrobial agent in ≥3 antimicrobial classes) using the criteria of Magiorakos and colleagues (14). In all cases, colistin was being considered as a treatment option for an infection caused by the isolate under investigation. For phase I, MIC testing was performed by three methods that were then used routinely in our laboratory on the 107 organisms at the time of isolation. Subsequently, 50 representative isolates (11 *A. baumannii*, 15 *K. pneumoniae*, and 24 *P. aeruginosa*) were selected for additional testing in phase II of the study based on availability and to include isolates with elevated colistin MICs. Prior to phase II testing, isolates were stocked in Brucella broth supplemented with 15% glycerol (BD Diagnostic Systems, Sparks, MD) at −70°C for ≤12 months and were subcultured two times prior to testing. Protocols were approved by the University of California, Los Angeles (UCLA), institutional review board.

Colistin susceptibility testing methods. In phase I testing, colistin MICs were determined by BMD with polysorbate 80 (BMD-T), broth/tube macrodilution (TDS), and the Etest on Mueller-Hinton agar (MHA) from BBL (BBL, BD Diagnostic Systems, Sparks, MD), in parallel, for each isolate. Per our laboratory protocol, water with 0.02% polysorbate 80 (BD Diagnostic Systems, Sparks, MD) was used to dilute the inoculum for BMD to obtain a final polysorbate 80 concentration of 0.002% in each well. During phase I and prior to storage at −70°C, the Etest on MHA from three manufacturers (BBL, Remel Scientific, Lenexa, KS, and Hardy Diagnostics, Santa Maria, CA) was also performed on the 50 representative isolates that would subsequently be examined in phase II. In phase II of testing, colistin MICs were determined for these 50 isolates in parallel by BMD-T, BMD without polysorbate 80 in the inoculum (BMD), AD, and Gram-negative extra MIC format (GNXF) TREK Sensititre panels. AD, BMD, and TDS testing was performed according to CLSI recommendations (12). Colistin susceptibility test methods are summarized in Table 1.

BMD panels were prepared in-house in untreated 96-well sterile polystyrene microplates (Evergreen Scientific, Los Angeles, CA). TDS was performed in borosilicate glass tubes washed with Micro-90 (International Products Corp., Burlington, NJ) prior to use for the preparation of colistin dilutions. For BMD, BMD-T, TDS, and AD, a 1,000-μg/ml stock solution of reagent-grade colistin sulfate (Sigma-Aldrich, St. Louis, MO) was prepared fresh in sterile deionized water. Incremental dilutions were made in either cation-adjusted Mueller-Hinton broth (CAMHB) (Difco, BD Diagnostics, Sparks, MD) for BMD, BMD-T, and TDS or Mueller-Hinton Agar (BBL) for AD. Two-fold dilutions of colistin concentrations tested ranged from 0.12 to 8.0 μg/ml for BMD and BMD-T, 0.06 to 16 μg/ml for TDS, and 0.25 to 16 μg/ml for agar dilution. Colistin concentration ranges included on Sensititre panels and Etest strips were 0.25 to 4.0 μg/ml and 0.016 to 256 μg/ml, respectively.

For all methods, 3 to 5 isolated colonies of an 18- to 24-h culture grown on 5% sheep's blood agar (BBL) were selected for testing. The Etest and Sensititre were performed according to each manufacturer's instructions. For reference BMD methods, standardized organism suspensions prepared in normal saline were further diluted in sterile water, or in sterile water containing 0.02% polysorbate 80 for BMD-T. Standardized suspensions were further diluted in CAMHB prior to the inoculation of TDS and in normal saline prior to the inoculation of AD. The final concentration of organisms tested by BMD, BMD-T, and TDS was approximately 3×10^5 to 5×10^5 CFU/ml, and that for AD was 10^4 CFU/spot. Tests were incubated for 16 to 20 h at 35°C in ambient air and were examined visually by two independent observers. For the Etest, a magnifying glass was used for the examination of zones. Quality control was assessed using the strains *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 (9).

Data analysis. A susceptible breakpoint of ≤2 μg/ml and a resistant breakpoint of ≥4 μg/ml were applied to all isolates. The CLSI intermediate breakpoint of 4 μg/ml for *P. aeruginosa* was not used for data analysis in this study (9). Colistin MICs obtained by BMD-T were used as the reference. This method was chosen because it is associated with the highest percentage of nominal colistin available in the test medium (K. Sei, presented at the meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing, Tempe, AZ, January 2012) (13). Essential agreement (EA) was calculated by the percentage of isolates with MICs within 1 doubling dilution from the reference method MIC. Categorical agreement (CA) was calculated by the percentage of isolates with MICs with the same categorical interpretation using all isolates tested as the denominator. VMEs were calculated using the number of resistant isolates as the

TABLE 2 Colistin MICs for subset of isolates that tested as nonsusceptible at time of isolation (phase I) and/or showed major or very major errors in either phase I or phase II with BMD-T as the reference method

Isolate no.[a]	Organism	MIC (μg/ml)[b]						
		Phase I (n = 107)			Phase II (n = 50)			
		BMD-T	TDS	Etest	BMD-T	BMD	AD	TREK
1*	*A. baumannii*	4	4	1[b]	0.5	8[c]	0.5	1
2	*A. baumannii*	8	16	1.5[b]	>8	>8	>16	>4
3	*A. baumannii*	>8	8	2[b]	>8	>8	8	4
4	*A. baumannii*	8	>16	3	>8	>8	>16	>4
5	*A. baumannii*	>8	16	0.5[b]	ND[d]	ND	ND	ND
6	*A. baumannii*	>8	16	3	ND	ND	ND	ND
7	*A. baumannii*	>8	16	12	ND	ND	ND	ND
7A	*A. baumannii*	2	4[c]	2	2	4[c]	2	1
8	*K. pneumoniae*	>8	>16	8	>8	>8	>16	>4
9	*K. pneumoniae*	>8	>16	12	>8	>8	>16	>4
10	*K. pneumoniae*	>8	>16	48	>8	>8	>16	>4
11	*K. pneumoniae*	>8	>16	0.5[b]	>8	>8	>16	>4
12	*K. pneumoniae*	>8	>16	8	>8	>8	>16	>4
13	*K. pneumoniae*	4	8	0.5[b]	8	2[b]	4	4
14*	*K. pneumoniae*	4	8	3	0.25	2	1	0.5
15*	*K. pneumoniae*	>8	>16	12	≤0.12	0.5	≤0.25	ND
16	*K. pneumoniae*	8	8	6	ND	ND	ND	ND
17*	*P. aeruginosa*	8	16	3	2	8[c]	4[c]	>4[c]
18	*P. aeruginosa*	2	1	3[c]	4	>8	2[b]	>4
19*	*P. aeruginosa*	4	4	4	2	4[c]	4[c]	2
20	*P. aeruginosa*	8	8	3	ND	ND	ND	ND
21	*P. aeruginosa*	0.25	1	4[c]	ND	ND	ND	ND
22	*P. aeruginosa*	0.5	0.5	3[c]	0.5	1	1	ND
23	*P. aeruginosa*	2	4[c]	2	2	4[c]	4[c]	2
24	*P. aeruginosa*	0.5	2	4[c]	ND	ND	ND	ND
25	*P. aeruginosa*	1	1	0.5	1	2	2	4[c]
No. of VMEs (%)			0 (0)	6 (32)		1 (10)	0 (0)	0 (0)
No. of MEs (%)			2 (2.3)	4 (4.7)		5 (12.5)	3 (7.5)	2 (5)
% EA			83	61		34	80	62
% CA			98	91		88	94	96

[a] Isolate numbers marked with an asterisk indicates isolates that lost resistance following storage and were discovered in phase II testing.
[b] Very major errors (VMEs) were calculated with 19 (phase I) or 10 (phase II) resistant (MIC, >2 μg/ml) isolates as the denominator.
[c] Major errors (MEs) were calculated with 88 (phase I) or 40 (phase II) susceptible (MIC, ≤2 μg/ml) isolates as the denominator. Essential agreement (EA) and categorical agreement (CA) were calculated with 107 (phase I) or 50 (phase II) isolates as the denominator.
[d] ND, not done, as isolates were unavailable for phase II testing.

denominator, and MEs were calculated using the number of susceptible isolates as the denominator (15). MICs obtained by the Etest that fell between 2-fold dilutions were rounded up to the nearest $\log_2$ value for these analyses so that BMD and Etest values were on the same scale. All VMEs and MEs were confirmed by repeat testing.

The significance of differences observed between test methods was calculated by Student's t test. P values of <0.02 were considered significant in this study.

RESULTS

Phase I. (i) Colistin MIC testing on 107 clinical MDR GNB by BMD-T, TDS, and Etest. Among 107 freshly isolated MDR GNB clinical isolates, 19 (17.8%) tested resistant to colistin by BMD-T. This included 7 *A. baumannii*, 9 *K. pneumoniae*, and 3 *P. aeruginosa* isolates. Fifteen isolates tested ≥1 dilution above the resistant breakpoint of 4 μg/ml (Table 2).

Two isolates tested susceptible by BMD-T but resistant by TDS (constituting an ME): one *P. aeruginosa* and one *A. baumannii* isolate, each with a susceptible MIC of 2.0 μg/ml by BMD-T but resistant at 4.0 μg/ml by TDS (Table 2). Eighty-three percent EA was found between BMD-T and TDS (Table 2). Sixty-one percent EA was found between BMD-T and the Etest (Fig. 1 and Table 2), and 6 VMEs were noted for the Etest (2 *K. pneumoniae* and 4 *A. baumannii* isolates). In particular, one *K. pneumoniae* and one *A. baumannii* isolate each tested with an MIC of 0.5 μg/ml by the Etest but >8.0 μg/ml by BMD-T (Table 2).

Interestingly, at susceptible concentrations <4.0 μg/ml in the 107 isolates, the colistin MICs measured by the Etest were significantly elevated compared to those measured by BMD-T (average MIC, 1.0 versus 0.5 μg/ml by BMD-T; $P < 0.0001$, Student's t test). However, at MICs of >4.0 μg/ml, Etest MICs were significantly reduced compared to BMD-T (average MIC, 6.5 versus 10.5 μg/ml by BMD-T; $P = 0.004$, Student's t test).

(ii) Effect of medium on Etest. In order to determine if the errors observed by the Etest were related to a particular medium source, colistin MICs were measured on MHA from three different commercial manufacturers, in parallel, for 50 representative isolates, including 15 colistin-resistant isolates. EA between the Etest and BMD-T was poor for all three sources of MHA and bacterial species, and was 46% on BBL, 64% on Hardy, and 68% on Remel MHA. However, CA was 78%, 78%, and 84% on each

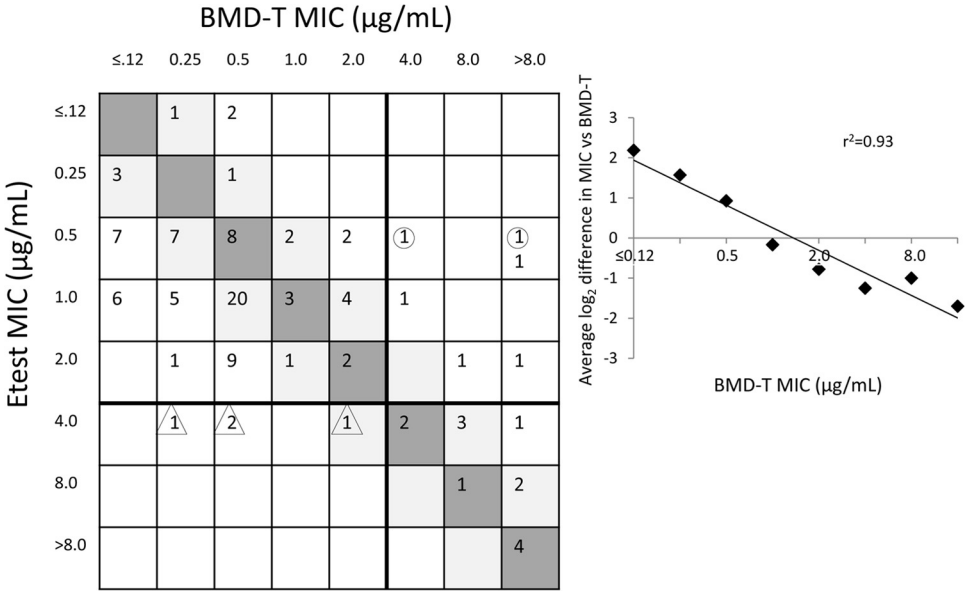

FIG 1 Scattergram of colistin MICs for 107 clinical isolates of MDR GNB (phase I), measured by BMD-T and the Etest on BD Mueller-Hinton agar. For isolates with major or very major errors, circles denote *K. pneumoniae* and triangles denote *P. aeruginosa.* Isolates of *A. baumannii* were not marked.

medium, respectively (Table 2). MHA from Hardy yielded one VME more than did media from the other two manufacturers, and that VME involved an *A. baumannii* isolate that tested resistant by BMD-T (Table 3).

Phase II. (i) Effect of polysorbate 80 on colistin BMD MIC testing. To determine the effect of surfactant in the BMD test on colistin MICs, a subset of 50 MDR GNB was tested in parallel by BMD, using either water or water with 0.02% polysorbate 80 as the inoculum diluent (BMD-T). Nine of 14 isolates available that tested resistant in phase I by BMD-T were resistant to colistin by BMD-T when retested in phase II, suggesting that 5 isolates lost the colistin-resistant phenotype following storage at −70°C. In 4 of the isolates (1 *A. baumannii*, 2 *K. pneumoniae*, and 1 *P. aeruginosa*), the reduction in MIC was >2 dilutions (Table 2). The fifth isolate, from *P. aeruginosa*, had a nonsusceptible MIC of 4 μg/ml when tested in phase I but had a susceptible MIC of 2 μg/ml in phase II. In addition, one *P. aeruginosa* isolate had a susceptible MIC of 2 μg/ml in phase I but tested resistant at 4 μg/ml in phase II (Table 2).

When tested in parallel, 34% EA was found between BMD and BMD-T MICs (Fig. 2 and Table 2). MICs measured by BMD were dramatically elevated compared to those measured by BMD-T ($P < 0.0001$, Student's *t* test; see Fig. 2). Sixty-two percent (31/50 isolates) had a >1-dilution-higher MIC by BMD than by BMD-T. Only one isolate, from *K. pneumoniae*, demonstrated >1-dilution-lower MIC by BMD versus BMD-T. The magnitude of the colistin MIC difference was inversely correlated with the MIC of each isolate obtained by BMD-T (Fig. 2 inset, $R^2 = 0.98$), such that larger-than-2-fold dilution differences in MICs were noted for isolates in the lower MIC range.

One VME was found by BMD testing in a *K. pneumoniae* isolate that had an MIC of 8.0 μg/ml by BMD-T but 2.0 μg/ml by BMD (Table 2). Five MEs were noted by BMD: 2 in *A. baumannii* and three in *P. aeruginosa* isolates (Fig. 2 and Table 2).

(ii) MIC testing by agar dilution. All 50 GNB were tested by AD as a second reference method. Eighty percent EA (40/50 isolates) was found between BMD-T and AD (Table 2). Three MEs were noted by AD compared to BMD-T for *P. aeruginosa* isolates (Table 2). One VME occurred in phase II for a *P. aeruginosa* isolate (Table 2), where the original BMD-T MIC was 2 μg/ml and on repeat in phase II was 4 μg/ml.

(iii) MIC testing by TREK Sensititre. MICs obtained by the TREK Sensititre panels were significantly higher than those obtained by BMD-T ($P = 0.07$, Student's *t* test; Fig. 3A) and no VMEs were observed (Fig. 3 and Table 2). Two MEs were observed, both in *P. aeruginosa* isolates (Fig. 3 and Table 2). Similar to what was noted with BMD, the differences in MICs obtained by

TABLE 3 Performance of the colistin Etest on MHA manufactured by BBL, Hardy, and Remel compared to results obtained with BMD-T during phase I testing

Organism	No. of isolates	No. of resistant isolates[a]	% CA[b]			% EA[b]			No. of MEs[b] (%)			No. of VMEs[b] (%)		
			BBL	Hardy	Remel	BBL	Hardy	Remel	BBL	Hardy	Remel	BBL	Hardy	Remel
A. baumannii	11	3	81	64	72	27	37	55	0	1 (12.5)	1 (12.5)	2 (66)	3 (100)	2 (66)
K. pneumoniae	15	9	80	80	80	33	67	66	0	0	0	3 (33)	3 (33)	3 (33)
P. aeruginosa	24	3	83	92	92	63	79	79	2 (9.5)	0	0	2 (66)	2 (66)	2 (66)
All isolates	50	15	78	78	84	46	64	68	2 (5.7)	1 (2.8)	1 (2.8)	7 (47)	8 (53)	7 (47)

[a] Isolates were designated resistant based on the MIC obtained by BMD-T. An MIC of ≥4 μg/ml was considered resistant.
[b] CA, categorical agreement; EA, essential agreement; MEs, major errors; VMEs, very major errors.

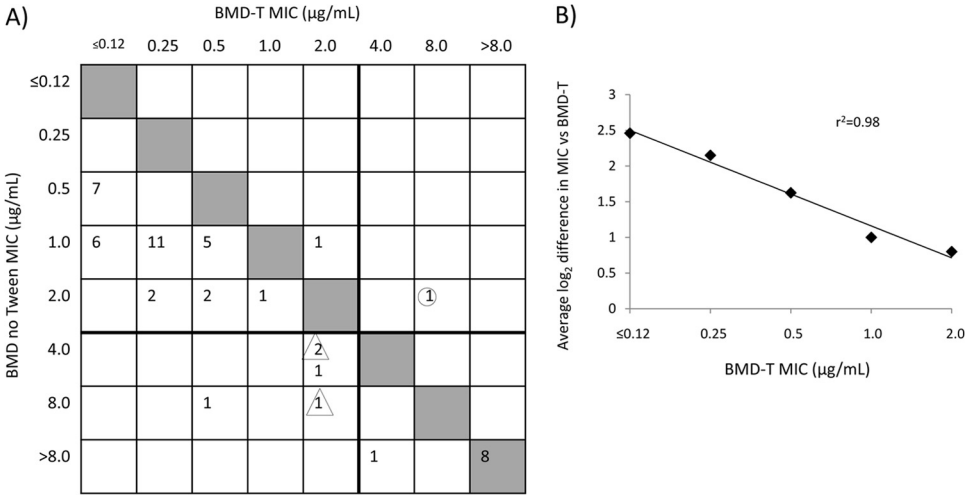

FIG 2 (A) Scattergram of colistin MICs for 50 MDR GNB (phase II), measured by BMD-T and BMD. For isolates with major or very major errors, circles denote *K. pneumoniae* and triangles denote *P. aeruginosa*. Isolates of *A. baumannii* were not marked. (B) Correlation between BMD-T MICs and the average number of $\log_2$ dilution changes in MIC when tested in the absence of polysorbate 80.

the TREK Sensititre versus BMD-T were most apparent at the low end of the MIC range, such that the greatest difference in MICs was noted among isolates with the lowest MICs by BMD-T (Fig. 3 inset, $R^2 = 0.91$).

QC testing results. The CLSI-recommended quality-control (QC) strains, *P. aeruginosa* ATCC 27853 and *E. coli* 25922, were tested by all methods. One hundred percent ($n = 27$) of the *P. aeruginosa* MICs were within the acceptable QC range of 0.5 to 4 μg/ml by all test methods. This is in contrast to MICs for *E. coli*, where 48.5% ($n = 27$) of the BMD-T, 44.5% ($n = 27$) of the Etest on BBL MHA, 47% ($n = 15$) of the Sensititre, and 3.8% ($n = 27$) of *E. coli* TDS MICs were below the acceptable range of 0.25 to 2 μg/ml.

DISCUSSION

In this study, we evaluated colistin MICs obtained by six test methods for a collection of contemporary MDR GNB isolated at our institution in 2010 and 2011. MICs obtained by BMD-T were used as the reference, although the inclusion of polysorbate 80 in BMD testing is currently not recommended by CLSI for testing colistin (12). The presence of polysorbate 80 is thought to allow for a better approximation of the true colistin MIC in that drug adsorption to polystyrene surfaces of BMD panels is minimized, and so a higher concentration of colistin is available in the broth to interact with the test bacteria (11, 13). In this study, we found a significant downward shift in colistin MICs measured by BMD with the addition of a 0.002% final concentration of polysorbate 80; this effect is most appreciable among organisms with lower MICs, a finding that also has been described by others (16–18). This is not altogether surprising, as one would anticipate the adsorption of colistin to surfaces to be the most observable at low concentrations of the drug. Karvanen and colleagues measured colistin concentrations in Mueller-Hinton broth following incubation in polypropylene, polystyrene, and glass tubes (11). After

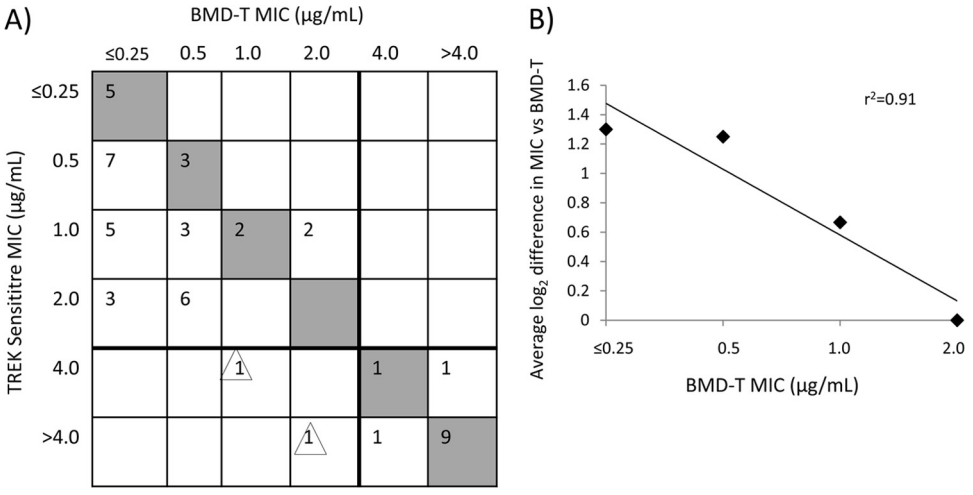

FIG 3 (A) Scattergram of colistin MICs for 50 MDR GNB (phase II) measured by BMD-T or the TREK Sensititre. For isolates with major or very major errors, circles denote *K. pneumoniae* and triangles denote *P. aeruginosa*. Isolates of *A. baumannii* were not marked. (B) Correlation between BMD-T MICs and the average number of $\log_2$ dilution changes in MIC when tested by TREK Sensititre.

24 h at 35°C, the measured starting concentration of 0.125 μg/ml colistin was only 8% of nominal in polystyrene (0.01 μg/ml), 13% in polypropylene (0.016 μg/ml), and 25% in glass (0.031 μg/ml) (11). In contrast, at a 4.0 μg/ml starting concentration, 75%, 62%, and 62% of nominal colistin remained following 24 h in polystyrene, polypropylene, and glass, respectively. Data presented at the January 2012 CLSI Antimicrobial Susceptibility Testing Subcommittee meeting demonstrated that the presence of 0.002% polysorbate 80 in CAMHB mitigated colistin adsorption to polystyrene microplates, such that a 64% loss occurs at a 0.5 μg/ml starting concentration with polysorbate 80, compared to a 92.5% loss in the absence of polysorbate 80, as measured by high-performance liquid chromatography. These observations correlate well with colistin MICs, and this is demonstrated in the results presented herein. The greatest 2-fold dilution increase in MICs when tested by BMD (Fig. 2) and the TREK Sensititre (Fig. 3) occurred among isolates with low MICs by BMD-T. When evaluated against BMD-T results, TDS had the highest EA and no VMEs (Table 2). Agreement of MICs obtained by TDS in glass tubes, which have the lowest colistin adsorption, with MICs obtained by BMD-T reinforces the role of colistin adsorption to polystyrene surfaces and the use of polysorbate 80 to mitigate this effect. However, it is important to note that the effect of polysorbate 80 on the relative viability of test organisms has not been fully evaluated. Nonetheless, inoculum water containing surfactant is used by some commercial manufacturers to aid in the dispersion of antimicrobial agents and organisms in antimicrobial susceptibility test systems. The addition of 0.002% polysorbate 80 is recommended for testing dalbavancin by BMD (19).

No VMEs were recorded for the TREK Sensititre, whereas 6 VMEs occurred with the Etest (BBL medium), representing 32% false susceptibility results among 19 resistant isolates (Table 2). Four of the Etest VMEs were found in 3 *A. baumannii* and 1 *K. pneumoniae* isolates with high MICs (>8.0 μg/ml) (Table 2). Interestingly, while the Etest MICs were significantly elevated compared to BMD-T MICs among susceptible isolates (e.g., those with MICs of <4.0 μg/ml), the Etest MICs were significantly lower than those obtained by BMD-T for resistant isolates. This might indicate poor diffusion of colistin through the agar medium, a problem that has been recognized for disk diffusion testing. Others have also reported poor performance of the Etest. In one study, 11% of the VMEs were observed among 15 colistin-resistant clinical isolates of *P. aeruginosa* (10) when agar dilution was used as the reference method. A second study of 25 *P. aeruginosa* isolated from cystic fibrosis patients reported 2 VMEs among 9 colistin-resistant isolates by the Etest as compared to agar dilution (6). Tan and Ng found 6.6% of the VMEs compared to BMD among 30 colistin-resistant *Enterobacteriaceae* (10). We questioned whether a medium effect might account for the high number of VMEs observed with the Etest; however, similar results occurred in testing on three different commercial manufacturers of prepared MHA (Table 3). All three media performed poorly.

A recent poll of clinical microbiology laboratories in the United States ($n = 66$ respondents) indicated that 44% rely on Etest results alone for colistin susceptibility testing, and another 13% use the Etest as a first-line test for colistin followed by a confirmation of the resistant results with a second method (R. M. Humphries, unpublished data). In contrast, only 15% of laboratories use AD or BMD for testing colistin. Numerous studies have demonstrated very good agreement between AD and BMD (4, 6, 20, 21), with the exception of *P. aeruginosa* isolated from cystic fibrosis patients, in whom AD might have more readily detected colistin resistance (5, 6). Among the 6 methods used in our study, we found the greatest EA between TDS (83%) and BMD-T, and between AD (80%) and BMD-T (Table 2). Given that media containing antibiotics that are required for these methods are not commercially available, neither TDS nor AD is practical for the routine clinical microbiology laboratory. While we found only 62% EA between the TREK Sensititre and BMD-T, categorical agreement was 96% and no VMEs were identified, suggesting that this method might be of use for clinical testing. However, the TREK colistin MIC test is labeled research use only (RUO).

A second pressing issue with colistin and polymyxin B testing is that the pharmacodynamics of colistin has not yet been fully defined (22). Recent data suggest that current CLSI and EUCAST breakpoints for colistin might be too high (22), as steady-state plasma concentrations on conventional dosing regimens are 1 to 2 mg/liter. As no clinical studies have investigated the correlation between colistin MICs and patient outcome, it is impossible to determine which method yields the most meaningful results at this time.

In this study, 5 isolates that initially tested resistant to colistin by BMD-T tested susceptible using this same method in phase II following 6 to 8 months storage in Brucella broth plus 15% glycerol at −70°C (Table 2). While the number of isolates tested was small, it suggests that colistin resistance might be lost following long-term storage. Loss of colistin resistance has also been documented to occur following the subculture of resistant isolates in the absence of selective pressure. Following just one passage in colistin-free medium, Li et al. documented the loss of the colistin-resistant phenotype in 98% of a colistin-resistant *A. baumannii* population (23). This finding might significantly impact clinical studies that evaluate patient outcome based on the MIC of each isolate, as retrospective testing to confirm MICs is frequently performed. Alternatively, this finding might indicate the presence of colistin heteroresistance, which has been documented in *A. baumannii* and *K. pneumoniae*, recovered both from patients treated with, and naïve to, colistin prior to isolation of the organism (24, 25). Finally, we noted 17.8% colistin resistance among 107 MDR GNB for which colistin was being considered as a treatment option at our facility during the study period. Surveillance studies have indicated <0.1 to 1.5% colistin resistance among contemporary GNB (26). However, it is clear that the incidence of colistin resistance might be significantly higher among MDR isolates, for which colistin might be used in treatment. In particular, in our study, a high rate (45%) of colistin resistance was observed among the 20 MDR *K. pneumoniae* isolates tested, an organism for which surveillance studies have noted only 1.5% resistance among all *K. pneumoniae* isolates examined (27). Others have also found high rates of colistin resistance in MDR and extensively drug-resistant *K. pneumoniae* (28–31). A retrospective chart review of the 19 patients with colistin-resistant isolates in our study revealed that none of them had documented colistin therapy in the 6 months prior to isolation of the organism (data not shown). These results, along with reports of community-associated colistin-resistant *Enterobacteriaceae* infections (32), underscore the need for a reliable reference method for colistin susceptibility testing. We suggest that BMD with polysorbate 80 should be the reference. The CLSI Subcommittee on Antimicrobial Susceptibility Testing has not yet defined a standard method for colistin testing, but in January 2013, quality control ranges for *E. coli*

ATCC 25922 and *P. aeruginosa* ATCC 27853 were accepted based on tests performed in CAMHB containing 0.002% polysorbate 80. This decision suggests that BMD with polysorbate 80 might become the reference method in the near future. Laboratories must remain cognizant of the variable results that can occur when different methods for colistin MIC testing are used.

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